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# Field efficacy of four anthelmintics and confirmation of drug-resistant nematodes by controlled efficacy test and pyrosequencing on a sheep and goat farm in Denmark

Miguel Peña-Espinoza<sup>a,b,\*</sup>, Stig M. Thamsborg<sup>b</sup>, Janina Demeler<sup>c</sup>, Heidi L. Enemark<sup>a</sup>

<sup>a</sup> Section of Bacteriology, Pathology and Parasitology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark

<sup>b</sup> Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Dyrslægevej 100, 1870 Frederiksberg C, Denmark

<sup>c</sup> Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, Königsweg 67, 14163 Berlin, Germany

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### ABSTRACT

We describe a case of anthelmintic resistance on one of the largest organic small ruminant farms in Denmark. The flock was established in 2007 by purchase of animals from other Danish farms and had history of clinical parasitism, high mortality of young stock and anthelmintic treatment failure. In October 2011, 40 lambs and 40 kids were selected for a faecal egg count reduction test (FECRT) with fenbendazole (FBZ), ivermectin (IVM), moxidectin (MOX) and levamisole (LEV). Lambs were treated with the recommended sheep dose of each product while kids received the sheep dose of IVM, 1.5× sheep dose of MOX and 2× sheep dose of FBZ and LEV. Untreated lambs and kids were also included and three methods for calculating faecal egg count (FEC) reduction were compared. In a subsequent investigation, a controlled efficacy test (CET) with FBZ and IVM was performed in lambs infected with *Haemonchus contortus* and *Trichostrongylus colubriformis* isolated from adult goats on the farm. Recovered specimens of *H. contortus* were subjected to pyrosequencing for detection of single nucleotide polymorphisms (SNPs) related to benzimidazole (BZ) resistance. During the FECRT, FECs in untreated lambs dropped significantly by 47%. No FEC reduction was detected in untreated kids. After FBZ treatments, FEC reductions in lambs and kids ranged from 15 to 54% and 49–56%, respectively, according to the different calculation methods. Post IVM treatments, FEC reductions in lambs and kids varied between 71–90% and 81–83%, correspondingly. LEV and MOX reduced FECs by 98–100% in both species. In the CET, FBZ reduced *H. contortus* worm counts by 52–56% and no reduction in *T. colubriformis* counts were detected after treatment. IVM eliminated 100% of *H. contortus* and reduced *T. colubriformis* counts by 84–92%, according to different calculation methods. Pyrosequencing of isolated *H. contortus* revealed increased frequencies of the BZ resistance-related SNP in codon 200 of the  $\beta$ -tubulin isotype 1 gene. Frequency of BZ resistance-related SNPs in codons 167 and 198 were very low and did not exceed levels as obtained in the

\* Corresponding author at: Section of Bacteriology, Pathology and Parasitology, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark. Tel.: +45 52612503; fax: +45 35886001.

E-mail addresses: [miap@vet.dtu.dk](mailto:miap@vet.dtu.dk), [miguelpenaespinosa@gmail.com](mailto:miguelpenaespinosa@gmail.com) (M. Peña-Espinoza).

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susceptible reference isolate. Anthelmintic resistance was confirmed in this recently established organic farm and low field efficacy of FBZ was verified by CET and pyrosequencing. BZ-resistant populations of *H. contortus* and *T. colubriformis* were isolated for the first time in Denmark. Problems with correct dosing of goats, the observed FEC reduction in untreated lambs and the relevance of including a control group in the FECRT are discussed.

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## 1. Introduction

In both, conventional and organic herds, preservation of the therapeutic efficacy of anthelmintics is vital for the treatment of heavily parasitized animals (Pomroy, 2006; Waller, 2006). Furthermore, effective anthelmintics are needed for the implementation of integrated parasite control programs which combine non-pharmacological methods with strategic use of drugs (Waller and Thamsborg, 2004). As a result, the monitoring of faecal egg count (FEC), the evaluation of treatment efficacy and the detection of anthelmintic resistance (AR) are becoming increasingly important for health programs of grazing livestock (Kaplan and Vidyashankar, 2012). AR is a problem usually associated with conventional production systems, which are more reliant on the use of antiparasitic drugs, but less in organic systems, where the prophylactic use of anthelmintics is banned (Waller, 2006; Hoste et al., 2014). In the present article we describe the investigations following a suspected case of AR in one of the largest organic sheep and goat dairy farms in Denmark. The objectives of our study were (1) to assess the efficacy of different anthelmintic treatments in animals naturally infected with gastrointestinal nematodes in the study farm, (2) to evaluate different methods for calculation of faecal egg count reduction percentages using untreated and treated lambs and kids and (3) to confirm the presence of suspected drug-resistant nematode strains in a controlled efficacy test. Additionally, benzimidazole (BZ) resistance was investigated using pyrosequencing analysis of the codons 167, 198 and 200 of the  $\beta$ -tubulin isotype 1 gene in nematode populations isolated in the controlled efficacy test.

## 2. Materials and methods

### 2.1. Study farm

The flock was established in 2007 by the purchase of animals from several Danish farms. No quarantine anthelmintic treatments were performed before introduction of new animals into the herd. At the start of the study (October 2011) it was one of the largest organic sheep and goat dairy herds in the country, including a total of 400 East Frisian sheep and 698 Saanen and Danish Landrace goats. Routinely, lambs and kids were weaned in April and managed as one group on common pastures. In early September young stock was moved to clean pastures. From the second grazing season onwards does and ewes grazed in separate groups, although using alternate grazing, on the same pastures. Until 2010, individual young stock with clinical signs of parasitism (<30% of the group per year)

were treated subcutaneously (s.c.) with macrocyclic lactones (ML), and affected adults (<10% of the group per year) were treated orally (p.o.) with BZ. In 2011, lambs and kids were weaned in late May. In early July several lambs and kids were observed with anaemia, diarrhoea, depression and suboptimal growth and animals with severe clinical signs (approximately 50% of the group) were treated with moxidectin (MOX, 0.2 mg/kg p.o., Cydectin® 0.1%, Scanvet). Roughly 25% of the animals were treated again with the same drug in mid-August. Clinical signs of parasitism continued in untreated animals and by mid-September the mortality reached approximately 25% in lambs and kids. Five lambs with diarrhoea and depression were submitted to the Large Animal Hospital, University of Copenhagen, and were treated with ivermectin (IVM) (0.2 mg/kg s.c., Ivomec® 1% Vet. injection, Merial Norden A/S), but no clinical recovery was observed. Hence, presence of AR was suspected.

### 2.2. Faecal egg count reduction test (FECRT)

In early October 2011 preliminary FECs of a group of lambs and kids at the study farm revealed a mean egg count of 4570 ( $\pm 3632$ ) eggs per gram (EPG) of faeces. From this group of animals, aged 4–6 months and not treated with anthelmintics within 4 weeks prior to the study, 40 lambs and 40 kids were selected. A FECRT was conducted following the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Coles et al., 1992). Individual body weights (BW) were obtained for all the animals using an electronic scale. Lambs and kids were stratified by FEC and randomly allocated to treatment groups ( $n=8$  animals/group, four groups for each species) and control groups ( $n=8$  animals/control group, one for each species). Lambs were treated with the recommended doses of fenbendazole (FBZ; 5 mg/kg p.o., Panacur® oral susp. 10%, MSD Animal Health), IVM (0.2 mg/kg s.c., Ivomec® 10 mg/mL Vet. injection, Merial Norden A/S), MOX (0.2 mg/kg p.o., Cydectin® mikstur 1 mg/mL, Scan Vet Animal Health A/S) or levamisole (LEV; 7 mg/kg s.c., Levaject® 75 mg/mL, ChemVet DK A/S). At the time of study, none of these anthelmintics were registered for use in goats in Denmark (VIF, 2011). Consequently, different doses were considered and kids were treated with FBZ (10 mg/kg p.o.), IVM (0.2 mg/kg s.c.), MOX (0.3 mg/kg p.o.) or LEV (14 mg/kg s.c.). The recommended dose of IVM for sheep (0.2 mg/kg s.c.) was maintained for kids based on previous experience by the authors of side-effects in goats treated subcutaneously with this drug. Faecal samples were collected rectally at the day of treatment (day 0) and at day 14 post treatment (p.t.) in all groups. After collection faeces was stored in ice-boxes at 5 °C, transported

to the laboratory and analysed within 24 h using a modified McMaster technique with a sensitivity of five EPG (Henriksen and Aagard, 1975; Henriksen and Korsholm, 1984). Larval cultures were prepared for every group (day 0 and day 14 p.t.) by mixing 10 g of faeces from each animal into one pool per group following the procedure described by Roepstorff and Nansen (1998). After baermannization, a minimum of 100 third stage larvae (L3) from each pool were identified at the genus level (MAFF, 1986) and the percentage of each nematode genus present was calculated.

### 2.3. Controlled efficacy test (CET)

In mid-January 2012, 3 months after the FECRT, faecal samples were collected from 20 adult goats naturally infected with gastrointestinal nematodes on the study farm. Fifty grams of faeces from each goat was mixed in a pool which was cultured to obtain infective L3 (Roepstorff and Nansen, 1998). Following baermannization, the number of harvested L3 per 1 mL was calculated on the basis of larval counts in  $10 \times 50 \mu\text{L}$  subsamples. The obtained population consisted of 67% *Trichostrongylus/Teladorsagia* spp. and 33% *Haemonchus contortus* L3 which were used to prepare infection doses of 9000 L3/animal. A CET was performed according to WAAVP guidelines (Wood et al., 1995). Eighteen indoor-reared, nematode-naïve Dorset lambs (aged 2–3 months) were challenged with the infective dose described above. At day 35 post infection lambs were stratified by body weight (BW; mean  $32 \pm 4$  kg) and randomly allocated into three groups of six lambs which were treated with FBZ (5 mg/kg p.o.), IVM (0.2 mg/kg s.c.) or left untreated (10 mL of water p.o.) to serve as control group. FECs were conducted in all lambs at the day of treatment (day 0) and day 7 p.t. using the modified McMaster technique described previously. At day 42 post infection (day 7 p.t.) all lambs were euthanized with a captive bolt pistol and exsanguinated. From each lamb the digestive tract was removed immediately and abomasums and small intestines were processed separately. Organs were opened into individual plastic buckets and the mucosa thoroughly washed with saline solution (0.9% NaCl) at  $38^\circ\text{C}$  until a total volume of 5 L was reached. Two 10% subsamples ( $2 \times 500$  mL) were taken from each organ and processed for recovery of motile adult nematodes using the agar-gel method (Christensen et al., 1995). All adult worms collected in each subsample were counted and the arithmetic means of both subsample counts were used to calculate the final worm count for each animal. Male worms were transferred to microscope slides for detailed examination. One drop of lactic acid (Sigma L1250, 10% v/v in distilled water) was added to clear the copulatory bursae and identification to species level was performed using the morphological keys reviewed by Barth (1991).

### 2.4. Pyrosequencing analysis

Pyrosequencing was performed in order to confirm the BZ resistance in *H. contortus* specimens recovered in the CET. Thirty adult male *H. contortus* isolated from FBZ-treated lambs at necropsy were subjected to DNA isolation using the Nucleo Spin Tissue Kit® (Machery & Nagel,

Germany) according the manufacturers protocol. In total six pools containing identical DNA amounts of five worms were prepared. Pyrosequencing was conducted in codons 167, 198 and 200 of the  $\beta$ -tubulin isotype 1 gene in order to detect single-nucleotide polymorphisms (SNPs) related to BZ resistance (Von Samson-Himmelstjerna et al., 2009). For each pool three replicates were performed. DNA obtained from L3 of a susceptible reference isolate (*H. contortus*, McMaster isolate) was used as for comparison. For the final results the arithmetic means of the results of each replicate were calculated.

### 2.5. Statistical analysis

In the FECRT, faecal egg count reductions percentages (FECR%) were calculated comparing three methods for calculation:

- (1)  $\text{FECR}\% = 100 \times (1 - [T2/C2])$ , (Coles et al., 1992)
- (2)  $\text{FECR}\% = 100 \times (1 - [T2/T1] \times [C1/C2])$ , (Dash et al., 1988)
- (3)  $\text{FECR}\% = 100 \times (1 - [T2/T1])$ , (Kochapakdee et al., 1995)

Arithmetic mean FECs of the treatment group at day 0 and 14 p.t. are indicated as T1 and T2, respectively; and C1 and C2 are arithmetic mean FECs of the control group at day 0 and 14 p.t., correspondingly. Using the methods by Coles et al. (1992) and Kochapakdee et al. (1995), AR is present if FECR % is less than 95% and the 95% lower confidence level is less than 90%. If only one of these conditions is fulfilled, AR is suspected. For the method by Dash et al. (1988), AR is present if FECR% is less than 80%. In order to identify differences in FECs of control groups during the study period, FECs were transformed ( $\log_{10}(x+1)$ ) and analysed using a paired *t* test. In the CET total worm counts of each nematode species were log-transformed for comparison between groups using a one-way ANOVA with a Bonferroni post hoc test. Efficacy of a given anthelmintic treatment to remove adult nematodes from the animals was calculated as worm count reduction percentage ( $\text{WCR}\% = 100 \times (1 - [T/C])$ ) (Wood et al., 1995; Bartley et al., 2004). Using the method of Wood et al. (1995), “T” is the geometric mean worm count of treatment groups and “C” the geometric mean worm count of the control group. According to Wood et al. (1995), an anthelmintic is highly effective if the WCR% is >98%, effective when the WCR% is 90–98%, moderately effective if WCR% is 80–89% and insufficiently effective if the WCR% is <80%. In the method described by Bartley et al. (2004), “T” and “C” are arithmetic mean worm counts in the treatment and control group, respectively, and resistance is present if WCR% is <95%. Statistical analyses were performed using the program Microsoft Excel® 2007 and Graph Pad® Prism (Version 5.1). A value of  $p < 0.05$  was considered significant.

## 3. Results

### 3.1. FECRT

Results of the FECRT in lambs and kids are presented in Tables 1 and 2, respectively. A significant reduction of

**Table 1**

Faecal egg count reduction test in lambs naturally infected with trichostrongyle nematodes on an organic small ruminant farm in Denmark, October 2011.

Treatment groups		Control (n = 8)	FBZ (n = 8)	IVM (n = 8)	MOX (n = 8)	LEV (n = 8)
Lambs	FEC Day 0 p.t. (±SEM)	3194 (±808)	2734 (±595)	4826 (±777)	3590 (±688)	1728 (±728)
	FEC Day 14 p.t.(±SEM)	1711 <sup>a</sup> (±442)	1250(±477)	501 <sup>b</sup> (±161)	0	22 <sup>b</sup> (±8)
	FECR %	–	27 <sup>R</sup>	71 <sup>R</sup>	100	99
	[95% CI] <sup>1</sup>	–	[–88–72]	[32–87]		[97–100]
	FECR % <sup>2</sup>	–	15 <sup>R</sup>	81	100	98
	FECR % <sup>3</sup>	47 <sup>R</sup>	54 <sup>R</sup>	90 <sup>R</sup>	100	99

FEC = arithmetic mean faecal egg counts; p.t. = post treatment; CI = confidence interval; SEM = standard error of the mean; FBZ = fenbendazole; IVM = ivermectin; MOX = moxidectin; LEV = levamisol.

<sup>1</sup> Coles et al. (1992).

<sup>2</sup> Dash et al. (1988).

<sup>3</sup> Kochapakdee et al. (1995).

<sup>R</sup> Anthelmintic resistance declared by the calculation method.

<sup>a</sup> Statistically different from EPG of control group day 0 ( $p < 0.05$ ).

<sup>b</sup> Statistically different from EPG of control group day 14 ( $p < 0.05$ ).

47% in the FECs of untreated control lambs was observed during the study period ( $p < 0.05$ ). No FEC reduction was detected in the untreated control group of kids. Treatment with FBZ reduced FECs of lambs and kids between 15–54% and 49–56%, respectively, depending on the different methods used for calculation. Accordingly, resistance to FBZ was declared by all three calculation methods in both animal species. At day 14 p.t., FECs of FBZ groups were not significantly different from FECs of control groups in lambs and kids. Larval cultures from animals at day 14 after FBZ treatment indicated a larval composition of 93% *Trichostrongylus/Teladorsagia* spp. and 7% *H. contortus* in lambs, and 100% *Trichostrongylus/Teladorsagia* spp. in kids. Treatment with IVM reduced the FECs of lambs and kids between 71–90% and 81–83%, respectively, based on the different methods used for calculation. Resistance to IVM was declared with two of the three methods in both lambs and kids. Larval cultures from IVM groups at day 14 p.t. indicated a larval composition of 92% *Trichostrongylus/Teladorsagia* spp. and 8% *Chabertia* spp. in lambs and 100% *Trichostrongylus/Teladorsagia* spp. in kids. LEV and MOX treatments reduced FECs of lambs and kids by 98–100% at day 14 p.t.

### 3.2. CEF

Faecal egg counts and worm burdens detected in lambs at the day of necropsy (Day 7 p.t.) are presented in Table 3. The *H. contortus* population was found to be highly resistant to FBZ (reductions of 52–56%) and fully susceptible to IVM (reduction 100%), using both arithmetic and geometric mean worm counts. The *Trichostrongylus colubriformis* population detected was not affected by FBZ treatment and worm counts were not statistically different from *T. colubriformis* burdens in the untreated control lambs ( $p > 0.05$ ). IVM reduced the number of adult *T. colubriformis* by 84–92%, depending on the method for calculation, and was significantly different from the adult *T. colubriformis* numbers in the control lambs ( $p < 0.05$ ). The *T. colubriformis* strain was confirmed fully resistant to FBZ and declared IVM-resistant using the method of (Bartley et al., 2004) but IVM-susceptible using the method of (Wood et al., 1995). Adult *Teladorsagia circumcincta* individuals were detected in the untreated control group with arithmetic and geometric mean worm counts of 37 and 34, respectively (data not shown). No *T. circumcincta* were detected in any of the treated lambs. The low number of *T. circumcincta* in the

**Table 2**

Faecal egg count reduction test in goat kids naturally infected with trichostrongyle nematodes on an organic small ruminant farm in Denmark, October 2011.

Treatment groups		Control (n = 8)	FBZ (n = 8)	IVM (n = 8)	MOX (n = 8)	LEV (n = 8)
Kids	FEC Day 0 p.t. (±SEM)	2404 (±351)	2150 (±682)	2708 (±646)	3774 (±2169)	3348 (±888)
	FEC Day 14 p.t. (±SEM)	2501 (±511)	1094 (±215)	484 <sup>a</sup> (±150)	3 <sup>a</sup> (±2)	14 <sup>a</sup> (±4)
	FECR%	–	56 <sup>R</sup>	81 <sup>R</sup>	100	99
	[95% CI] <sup>1</sup>	–	[22–76]	[59–91]		[99–100]
	FECR % <sup>2</sup>	–	51 <sup>R</sup>	83	100	100
	FECR % <sup>3</sup>	0	49 <sup>R</sup>	82 <sup>R</sup>	100	100

FEC = arithmetic mean faecal egg counts; p.t. = post treatment; CI = confidence interval; SEM = standard error of the mean; FBZ = fenbendazole; IVM = ivermectin; MOX = moxidectin; LEV = levamisol.

<sup>1</sup> Coles et al. (1992).

<sup>2</sup> Dash et al. (1988).

<sup>3</sup> Kochapakdee et al. (1995).

<sup>R</sup> Anthelmintic resistance declared by the calculation method.

<sup>a</sup> Statistically different from EPG of control group day 14 ( $p < 0.05$ ).



**Table 3**

Faecal egg counts and worm numbers of adult *H. contortus* and *T. colubriformis* in lambs 7 days post treatment in a controlled efficacy test.

Treatment group	FEC at necropsy (epg)	<i>H. contortus</i> adult worm counts				<i>T. colubriformis</i> adult worm counts			
	AM ( $\pm$ SEM)	AM ( $\pm$ SEM)	GM	% WCR <sup>1</sup>	% WCR <sup>2</sup>	AM ( $\pm$ SEM)	GM	% WCR <sup>1</sup>	% WCR <sup>2</sup>
Control (n = 6)	2103 ( $\pm$ 344)	269 ( $\pm$ 41)	255	–	–	833 ( $\pm$ 182)	738	–	–
FBZ (n = 6)	724 ( $\pm$ 179)	128 ( $\pm$ 66) <sup>a</sup>	113 <sup>a</sup>	56	52	1194 ( $\pm$ 141)	1157	0	0
IVM (n = 6)	10 ( $\pm$ 5)	0 <sup>a</sup>	0 <sup>a</sup>	100	100	136 ( $\pm$ 174) <sup>a</sup>	57 <sup>a</sup>	92	84

FEC = faecal egg count; epg = eggs per gram of faeces; AM = arithmetic mean; GM = geometric mean; WCR = adult worm counts reduction; SEM = standard error of the mean.

<sup>1</sup>Wood et al. (1995).

<sup>2</sup>Bartley et al. (2004).

<sup>a</sup> Statistically different from control group ( $p < 0.05$ ).

control group was far below the minimum 100 worms required for assessment of anthelmintic efficacy (Wood et al., 1995).

### 3.3. Pyrosequencing

Allele frequency analyses of the SNPs related to BZ resistance in 30 adult male *H. contortus* isolated in the CET are presented in Table 4. Frequencies of the SNPs associated with BZ resistance detected in codons 167 (TTC to TAC) or 198 (GAA to GCA) were very low with mean values between 0.3 and 2%, which can be considered as background. In contrast, frequencies of the allele associated with BZ resistance in codon 200 (TTC to TAC) were significantly increased with values between 96 and 99%. The BZ-susceptible control isolate displayed SNP frequencies between 1 and 4% in all three codons.

## 4. Discussion

The presence of AR on the investigated farm was initially declared by FECRT and subsequently confirmed by CET and pyrosequencing. In the FECRT, FBZ treatments were markedly ineffective in reducing FECs in both lambs and kids and resistance was indicated by all three calculation methods. IVM resistance was also declared in both animal species by two of the three methods for calculation of FEC reduction. This is in accordance with early reports from Denmark indicating reduced field efficacy of BZ and IVM in sheep and goat farms (Bjørn et al., 1991; Maingi et al., 1996, 1997). In contrast, MOX and LEV were highly effective in the FECRT. LEV had never been used before in the study farm and MOX was the drug of choice for individual treatments in 2011. Reduced LEV field efficacy was previously reported in Danish sheep (Bjørn et al., 1991; Maingi et al., 1997) and goat farms (Maingi et al., 1996). Although no studies have ever been published on the field efficacy of MOX in Denmark, resistance against this drug in small ruminant nematodes has been confirmed in Europe (Sargison et al., 2010). Moreover, studies in cattle suggest cautious interpretation of MOX efficacy obtained from FECRT due to inconsistent results between FECRT and CET (Condi et al., 2009; De Graef et al., 2012). Condi et al. (2009) reported a

FEC reduction of 92% but a worm count reduction of 65% in *Cooperia* spp. and 45% in *Oesophagostomum radiatum* in steers, 14 days p.t. with subcutaneous MOX. Using experimentally infected calves, De Graef et al. (2012) observed a FEC reduction of 86% but a worm count reduction of 31% in *C. oncophora*, 14 days after subcutaneous MOX treatment. These findings were explained by a temporary suppression of the egg excretion by female nematodes following MOX treatment and it illustrates that a high FECR% after MOX treatment is not necessarily correlated with complete elimination of the nematode population from the host. Accordingly, it has been suggested that FECs should be analysed, at minimum, 17 days after MOX treatment in a FECRT (Condi et al., 2009; Kaplan and Vidyashankar, 2012).

As regards the three methods selected to calculate FEC reduction, the major difference between them is whether or not they include an untreated control group. In our study, a statistically significant FEC reduction of 47% was detected between day 0 and 14 p.t. in untreated control lambs. If this reduction remains unnoticed, i.e. if no untreated controls are included, this could lead to an apparent increase in the efficacy of a given drug, thereby decreasing the sensitivity of the FECRT. Accordingly, by using the method in which no untreated control group is incorporated (Kochapakdee et al., 1995), FBZ and IVM treatments in lambs achieved their highest efficacies compared to the other two methods. In contrast, no mean FEC reduction was observed in untreated control kids during the study period, and similar efficacies were obtained after FBZ and IVM treatments using all calculations methods. The observed FEC reduction in untreated lambs may be explained by the development of acquired immunity in these animals, leading to expulsion or a reduced fecundity of female nematodes, as reported by Barger (1988) and Dobson et al. (1990). In contrast, it is known that goats do not develop effective immunity against gastrointestinal nematodes during the first 12 months of age (Hoste et al., 2010), which can explain why FECs in the untreated control kids remained steadily high throughout our study. The inclusion of untreated animals in the FECRT has been recommended in order to detect natural FEC variation during the study period allowing for corrections in the efficacy estimation of drugs in the treated group (Presidente, 1985; Coles et al., 1992;

**Table 4**

Mean allele frequencies of the single-nucleotide polymorphisms (SNPs) associated with benzimidazole-resistance in codons 167, 198 and 200 in the  $\beta$ -tubulin isotype 1 gene in *H. contortus* determined by pyrosequencing.

<i>H. contortus</i> (n = 5 worms/pool)	Frequency of SNPs in $\beta$ -tubulin isotype 1 gene		
	Codon 167 (TAC)	Codon 198 (GCA)	Codon 200 (TAC)
Pool 1	0.3	2.0	98.3
Pool 2	2.3	2.6	96.7
Pool 3	2.0	1.8	94.0
Pool 4	1.7	3.3	97.8
Pool 5	1.0	2.7	99.2
Pool 6	3.0	1.5	98.5

Lyndal-Murphy et al., 2014). McKenna (2013) suggested that these corrections are particularly relevant when an ineffective anthelmintic is being evaluated, but less important if a drug is highly efficient. This is in agreement with our findings of higher variation in FECR%, after correction, in FBZ and IVM treated animals, compared to less variation in animals treated with the highly efficient MOX and LEV. Based on computational modelling, Dobson et al. (2012) claimed that no evidence exists to indicate that studying the FEC variation of untreated animal enhances the efficacy evaluation of an anthelmintic in a FECRT. In contrast, and also based on computer simulation, Lyndal-Murphy et al. (2014) recommended to include untreated controls in the FECRT, particularly if the infection pressure is high and an increase of FEC is expected during the study. In practice, the inclusion of untreated controls is expensive, laborious and often unwanted by the farmer. In our study inclusion of an untreated group and use of different calculation methods did not substantially affect the declarations of anthelmintic efficacy in the FECRT (i.e. strong BZ resistance and high efficacy of MOX and LEV) with the exception of IVM that was declared effective only with the method of Dash et al. (1988), which includes a control group, and subsequently confirmed by the good efficacy of IVM in the CET. However, the inclusion of untreated controls, if feasible, allows a wider range of calculations and corrections of efficacy estimations, especially when studying a nematode population of unknown susceptibility to different drugs, as observed in our study.

For the performance of FECRTs in sheep and goats oral anthelmintics should preferably be used at the recommended dose rate and route (Coles et al., 1992, 2006). In the present study oral formulations of FBZ and MOX were used in lambs and kids. Whereas subcutaneous IVM and LEV treatments were applied to both animal species as no oral formulations of these two drugs were registered for ruminants in Denmark at the time of the study (VIF, 2011). All anthelmintics in lambs were administered using the recommended dose rate for sheep. However, none of the anthelmintics used in the study were registered for use in goats in Denmark (VIF, 2011). Therefore, higher doses were calculated for kids as suggested by several scientists (Silvestre et al., 2002; Lespine et al., 2012). Thus, kids were treated with 2 $\times$  sheep dose rates of FBZ and LEV and 1.5 $\times$  sheep dose rate of MOX. For IVM, the recommended sheep dose (0.2 mg/kg) was used in kids based on experience by the authors of undesirable side-effects in goats

treated subcutaneously with this drug. Facing a fully IVM-susceptible nematode population, it has been suggested that the rapid metabolism of IVM in goats might not cause a lower efficacy (Gopal et al., 1999). Yet, against worm populations with emerging IVM resistance a lower dosage in goats could result in reduced anthelmintic efficacy and further selection for resistance (Gopal et al., 1999).

Following the FECRT, a CET was conducted in order to identify the nematode species contributing to the suspected BZ and IVM resistance indicated in the field. Attempts were made to perform post-mortem examination of lambs and kids previously treated in the FECRT, however, the farmer was unwilling to sell any of these animals. Yet, the isolation in the CET, post FBZ treatment, of populations of *H. contortus* (low FBZ efficacy) and *T. colubriformis* (nil FBZ efficacy) confirmed the presence of BZ resistance in the farm. In comparison, IVM treatment effectively removed the total *H. contortus* adult population while *T. colubriformis* was not completely eliminated from the infected lambs. Using arithmetic mean worm counts and the threshold for efficacy described by Bartley et al. (2004), the *T. colubriformis* population was found to be IVM resistant. However, using geometric mean worm counts and the interpretation of Wood et al. (1995), IVM had a moderate effect against *T. colubriformis*. Consequently, the presence of IVM resistance in this *T. colubriformis* population is not clear and should be further assessed by comparing its susceptibility to IVM administered both orally and subcutaneously. Previous studies have demonstrated a reduced efficacy of IVM administered subcutaneously against *T. colubriformis* in sheep (Giordano et al., 1988) and cattle (Egerton et al., 1981) and against *Trichostrongylus* spp. infections in goats (Pearson and Rutherford, 1988), when compared with oral IVM drenching. Giordano et al. (1988) reported that *T. colubriformis* worm counts were reduced by 85% in lambs following subcutaneous IVM treatment and by >99% after intra-ruminal administration, using an identical dose of 0.2 mg IVM/kg BW. In goats infected with *Trichostrongylus* spp., the same IVM dose reduced FECs by 94% and 100% using subcutaneous and oral routes, respectively (Pearson and Rutherford, 1988). Similarly, in calves infected with *T. colubriformis* a worm reduction of 90% was seen after subcutaneous treatment whereas >95% of the worms were removed after oral IVM treatment using the same dose (Egerton et al., 1981).

Pyrosequencing analysis of adult *H. contortus* isolated after FBZ treatment in the CET confirmed the resistance to

BZ, with highly increased frequencies of the allele correlating with resistance in codon 200 of the  $\beta$ -tubulin isotype 1 gene.

For comparison, a parallel pyrosequencing was performed with DNA from a L3 of a BZ-susceptible *H. contortus* isolate (McMaster isolate), instead of worms from untreated control lambs in the CET. Nonetheless the susceptible isolate corresponds to a different strain, it is unlikely that worms from the untreated animals would have had significantly lower SNP frequencies than this BZ-susceptible reference isolate, considering that the study population was strongly selected for BZ-resistance, as indicated by the high frequencies observed at codon 200. These high frequencies detected in codon 200 and the very low frequencies of SNPs related to BZ resistance in codons 167 and 198 in the same gene is congruent with results of other studies assessing allele frequencies in BZ-resistant *H. contortus* (Von Samson-Himmelstjerna et al., 2009; Barrère et al., 2012, 2013). Currently there is no pyrosequencing assay available to investigate these SNPs in *T. colubriformis* and this was not intended in the present study.

Finally, the high level of BZ resistance detected only 4 years after the establishment of the herd could possibly be explained by the introduction of resistant worms with purchased stock. Since the establishment of the farm selective anthelmintic treatment has primarily been directed against individual animals with clinical signs of parasitism, and thus the selection pressure for development of AR is considered to have been minimal. Quarantine treatments of purchased animals have been promoted to avoid the introduction of AR into farms (Coles and Roush, 1992; Kaplan and Vidyashankar, 2012; Leathwick and Besier, 2014) but this may not be acceptable in organic production systems unless animals with positive FEC are confirmed. Highly efficacious drugs or a combination of anthelmintics should be used in quarantine treatments, with the subsequent confirmation of a negative FEC before turn-out to pasture. Thus, the success of this approach relies on the availability of sensitive and accurate diagnostic methods.

## 5. Conclusions

A suspected case of AR in a large organic sheep and goat farm was studied by FECRT, CET and pyrosequencing analysis for *H. contortus*. BZ and IVM resistance were detected by FECRT but only BZ resistance was fully confirmed by CET and pyrosequencing. The significant natural reduction of FECs in the untreated lambs during the FECRT emphasizes the relevance of including a control group to ensure precise determination of drug efficiency. The finding of BZ resistance in *T. colubriformis* and the highly pathogenic *H. contortus* in a recently established organic herd demonstrates the need for close surveillance of purchased stock to avoid introduction of resistant nematodes.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

MPE, SMT and HLE designed the study and performed anthelmintic treatments, faecal sampling and post-mortem examinations. MPE carried out laboratory and statistical analysis and drafted the manuscript. JD performed pyrosequencing of the specimen recovered within the CET. All authors provided contribution to the manuscript and approved the final version.

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